



Faculty of Resource Science and Technology

## **PROTEIN PROFILING OF DISEASE *Piper nigrum***

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## **Protein Profiling of Diseased *Piper nigrum***

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## Declaration

I hereby declare that the thesis entitled “ Protein Profiling of Disease *Piper nigrum*” submitted here with and for Bachelor of Degree with Honours in Resource Biotechnology at University Malaysia Sarawak as my own work (except for the cited references) and has not been previously submitted by me at any other University for any purposes.

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Date : 18/8/14



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## List of Abbreviations

°C	Degree celcius
μl	microlitre
2-DE	2-dimensional gel electrophoresis
BSA	Bovine serum albumin
d H <sub>2</sub> O	Distilled water
EDTA	Ethylenediaminetetraacetic acid
g	Gram
KCl	Potassium chloride
kDa	Kilo Dalton
min	Minute
ml	Millilitre
ng	Nanogram
PMSF	Phenylmethylsulfonyl fluoride
PR	Pathogenesis Related
PTM	Posttranslational modification
rcf	Relative centrifugal force
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate -Polyacrylamide gel electrophoresis
TEMED	Tetramethylethylenediamine
β- ME	Beta-mercaptoethanol

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## Abstract

*Phytophthora capsici* is the main cause of the foot rot disease affected the *P. nigrum* plant. As the disease can cause economic loss to the agriculture, this research was performed to analyse the existence of any distinct protein between healthy and the diseased plant of the *P. nigrum* from the roots sample. Two methods of protein extraction were used to identify the difference of protein yield and better quality with quantification of protein using two assays which were Bradford assays and BCA assays. The SDS-PAGE analysis had revealed two different unique proteins band of molecular weight ~25 kDa and ~11 kDa from the diseased samples. Metaproteome has the potential to discover the new proteins contributing to analysis of pathogenesis related proteins. At the end of the experiment, the proteins were successful extracted from the roots of the infected plant with new protein profile discovered showing difference between the diseased and non-diseased *P. nigrum*.

Keywords: *Piper nigrum*, *Phytophthora capsici*, proteomic analysis, metaproteome

## Abstrak

*Phytophthora capsici* merupakan penyakit utama yang mempengaruhi pertumbuhan pokok lada hitam. Penyakit ini menyebabkan kerugian ekonomi dalam bidang pertanian dan kajian ini telah dilakukan untuk menganalisis perbezaan yang wujud dalam protein antara pokok yang sihat dan pokok yang dijangkiti penyakit melalui akar pokok lada hitam. Dua kaedah untuk memisahkan protein daripada akar telah digunakan untuk mengenal pasti perbezaan protein yang diperolehi dan dua kaedah ujian pengkuantitian protein turut dijalankan iaitu Bradford dan BCA assay. Analisis melalui Elektrofesis gel poliakrilamida-Sodium Dodesil Sulfat telah mendapati dua jalur protein yang berbeza daripada sampel akar yang dijangkiti dengan berat molekul ~25 kDa dan ~11 kDa. Metaproteom pula berpotensi untuk mengenal pasti protein yang baru yang boleh menyumbang kepada analisis protein berkaitan dengan patogenesis. Pada akhir kajian ini, protein telah berjaya dipisahkan dan protein yang baru telah ditemui daripada sampel yang berpenyakit dan perbezaan antara pokok lada hitam yang sihat dan berpenyakit telah dapat dikenal pasti.

Kata kunci: Lada hitam, *Phytophthora capsici*, proteomik analisis, metaproteom.

## 1.0 Introduction

*Piper nigrum* plant known as black pepper is a type of woody perennial climbers and rarely shrub with dilated or swollen nodes and stipule. Peppers from Indian origins are widely used internationally such as in Thailand and Malaysia because of high quality and produce more fruits. Globally, the berry part had been used as spices and seasoning due to its pungent flavour and increased aromatic scent in foods. While, different parts of the plant had been used as secondary metabolites in drugs, preservative, insecticidal and larvicidal control agents (Ahmad *et al.*, 2012). Recent studies by Srinivasan, had shown that *P. nigrum* have effects on digestion system where piperine increase the production of saliva and gastric secretions (Srinivasan, 2007). The digestive system produce by the ingestion of *P. nigrum* probably help to stimulate the liver to secrete bile which further digests food substances with enhancing the digestive capacity and significantly reduces the gastrointestinal food transit time.

This research will focus on the roots parts of the *P. nigrum* to identify the different proteins between the healthy and disease roots of the plants for the proteomic studies. This is because, the studied from leaves parts, had revealed the role of different pathogenesis related (PR) proteins and enzymes in the disease reaction of black pepper with respect to *Phytophthora capsici* foot rot infection (Nazeem *et al.*, 2008). Mostly, the proteomic studies had developed widely to allow the quantitative and qualitative separation of complex protein mixtures, which makes it possible to monitor dynamic protein expression in plant growth and metabolism by using two-dimensional electrophoresis (2-DE) and mass spectrometry (Ma *et al.*, 2013). Recent studies from Zeng *et al.* have identified the

proteomic changes in apple trees during their vegetative stages by 2-DE and mass spectrometry (Zeng *et al.*, 2010). The proteomics also identify new proteins and abundance of differential amino acids from the plant parts, thus further research can be conducted for the researchers to allocate the benefits in medicinal value.

The hypothesis of the research is to identify any unique protein existed between the roots of healthy and diseased *P. nigrum* plant. There are few objectives implemented in the study which to extract the protein from the roots of normal and infected *P. nigrum* plant. This was conducted in triplicates to get an average data of protein concentration. Two different method of extraction used for this proteomic studies and subsequently another goal is to identify the best method for the protocol which yield more protein. Phenol extraction method and phenol/SDS extraction method are chosen in the protocols which are differing from the use of chemical ingredient and certain steps. Moreover, the study also focused in comparing the protein quantification method that yields more protein concentration between Bradford assays and Bicichinonic acid (BCA) assays. Another objective is to differentiate and visualize the unique marker of the protein using one dimension gel electrophoresis and then compare the staining method of the gel using Comassie Brilliant Blue (CBB) staining and silver staining.



## 2.0 Literature Review

### 2.1 *Piper nigrum*

*Piper nigrum* species had been used widely as preserving, insecticidal material and commercially in medicine from different parts of the plant. *P. nigrum*, *P. longum* and *P. betle* is the most popular species from more than 1000 species genus piper had reported by the study (Ahmad *et al.*, 2012) and had proved the used as anti-apoptotic, antibacterial, anti-colon toxin, antidepressant, antifungal, antidiarrheal, anti-inflammatory, anti-mutagenic, anti-metastatic activity, anti-oxidative, antidiuretic, antispasmodic, antispermatic, antitumor, anti-thyroid, ciprofloxacin potentiator, cold extremities, gastric ailments, hepatoprotective, insecticidal activity, intermittent fever, and larvicidal activity from the peppercorn and secondary metabolites. The fruits of this plant can produce white pepper, green pepper and black pepper and its active component of piperine help to stimulate the enzyme in the digestive systems of pancreas and intestines. In medicine, black pepper shows main role to cure digestive disorders diseases such as large intestine toxins, gastric problems, diarrhea and indigestion, therefore, also for the treatments to fight respiratory disorders which include cold, fever and asthma.

The origin of *P. nigrum* is from the South India and developed extensively through tropical regions. In 2008, Vietnam becomes the world's largest producer and exporter of pepper crops. It requires moist, hot and tropical climate from sea level up to approximately 600 m elevation. Furthermore, evenly distributed annual rainfall about 2 500 or more and

soils rich in humus with acidic pH between 5.5 to 6.0 become necessary for the growth of the plant (Cannon-Eger, 2011).

Besides, the study on *P. nigrum* plants regarding the expression of pathogenesis related proteins in the plants which mostly related foot rot diseases using leaves parts had revealed two distinct proteins band from diseased leaves by SDS-PAGE analysis. While the study on RT-PCR for the detection of *Cucumber mosaic viruses* and *Piper yellow mottle virus* indicated the presence of both viruses that associated with stunt diseased of *P. nigrum* (Bhat & Siju, 2007; Nazeem *et al.*, 2008).

## **2.2 *Phytophthora capsici***

Recent studies had shown that the foot rot is the serious disease that had occurs on the root of black pepper plant that cause by *Phytophthora capsici*. *P. capsici*, is characterized as a soil-born fungus which causes severe crop losses due to the infection of the plant (Nazeem *et al.*, 2008). Generally, the plants fight the pathogens occur such as bacteria, fungi or viruses by accumulating a number of specific proteins in the intercellular spaces that are known as Pathogenesis Related (PR) proteins and associated with the defence mechanism. The most extensively studied PR proteins include chitinase and  $\beta$ -1,3 glucanase, as they are known to inhibit the fungal growth by lysing the fungal cell walls.

Proteomics developed as an influential tool for the study of plant's response to many level of stress factors, including plant pathogen interactions, plant-herbivores interactions and wounding. The proteomic analysis stage helps to estimate the abundance of proteins in the tissue. The basics method used in proteomics studies is 2-D gel electrophoresis and mass spectrometry. It is used for separating and identifying proteins at the proteome level (Cho *et al.*, 2006). Both provides a reasonably good resolution and coverage of the proteome and is particularly useful for the identification of isoforms and PTM (Ma *et al.*, 2013).

Both of the method allow the quantitative and qualitative separation of complex protein mixtures, which makes it possible to monitor dynamic protein expression in plant growth and metabolism (Ma *et al.*, 2013).Recent studies have successfully compared the different growth stages of rice using the proteomics method to expound the roles of expressed proteins over the course of growth (Nozu *et al.*, 2006) and have identified the proteomic changes in apple trees during their vegetative stages by 2-DE and mass spectrometry (Zeng *et al.*, 2010).

## 2.4 Metaproteome

Protein composition in natural microbial community or metaproteome is expressed in application of proteomics approaches that is called metaproteomic. The protein analysis is influence in the roots sample because there is another living microorganism from the soil land. In another word, analysis of metaproteome in ecology should allow new tracking of functional genes, metabolic pathways and identifying proteins linked with specific stresses

(Maron *et al.*, 2007). The recent research from Kan *et al.*, 2005, had developed the first metaproteomic study of aquatic microbial assemblages and demonstrate the potential of metaproteomic approaches to link metagenomic data, taxonomic diversity, functional diversity and biological processes in natural environments (Kan *et al.*, 2005). Furthermore, a direct measurement of functional gene expression in metaproteomics can also be express in terms of the presence, relative abundance and modification state of proteins (Wilmes & Bond, 2004).

## **2.5 Bradford assays**

General technique or the common technique to quantify the total protein concentration is by using Bradford assays. Other than that, there are also several techniques for total protein quantification such as 1- Biuret method, which well-known as an old method, 2- variation of Lowry method, 3- Bicinchinonic acid (BCA) assays, 4- Acid hydrolysis, 5- Ultraviolet spectrophotometry, and 6- dry weight measurement. The Coomassie dye binding or known as Bradford assay is a quite simple and easy. It is frequently quite sensitive, but, it also gives variable responds depending on how well or how poor the protein binds with the dye in acid pH (Lovrien & Matulis, 1995). If the binding of the protein with the dye is poor, it will give low absorbance value and vice versa. When the dye bind to the proteins, the colour of the solution change that indicates a measure of total protein, which is quite sensitive in the case of albumin and certain globular proteins (Bradford, 1976). The Bradford reagent can be prepared by using Coomassie Brilliant Blue (CBB) G-250, with the mixture of methanol (100%), phosphoric acid (85%) and deionized water (d H<sub>2</sub>O). The absorbance value can be measured at range 365 nm to 595 nm.



## 2.6 Bicinchoninic acid (BCA) assays

The BCA assays for total protein gives different sensitivity compare with Coomassie dye binding assays. It is more sensitive in response to incubation time, incubation temperature, standard protein used for calibration and other factors (Smith *et al.*, 1985). This assay is based on the alkaline reduction of the cupric ion to the cuprous ion by the protein, followed by chelation and colour development by the BCA reagent which produce purple colour as the end product. There also some classes of interfering compounds such as reducing sugars and ammonium ions interfere with the assays, however, the assays will have a good combination of sensitivity and simplicity if the interfering compounds are eliminated properly (Lovrien & Matulis, 1995). In addition, the reagent of BCA assays is fairly stable under alkaline conditions and can be included in the copper solution to allow one step procedure. Two reagent are divided that include Reagent A and Reagent B. Reagent A are consists of sodium bicinchoninate, sodium carbonate, sodium tartrate, sodium hydroxide and sodium bicarbonate with the proper measure to mix with Reagent B which is cupric sulphate solution. When both reagent A and B are mix together with 100: 2 volumes, the blue-green (emerald) colour are visible as the standard working solution (Stoscheck, 1990). The absorbance reading is measure at 562 nm.

## 2.7 Silver staining

Polyacrylamide gel electrophoresis (PAGE) is a commonly used technique for analysis of proteins because of its low cost, ease of use, and high sensitivity with one-dimensional or two-dimensional gel analysis. The proteins mixture then are visualized after run the PAGE

gel, usually by using Coomassie Brilliant Blue (CBB) R (Schuchard *et al.*, n.d.). However, there is another method for staining the gel which is 10-100 fold increase sensitivity than the general staining. It is known as Silver staining method. Silver staining can detect as low as 0.1-1 ng protein on the gel comparing with the CBB R staining, it only detect a 50 ng protein band. The silver staining had developed into various concepts since 1979, where the detection levels of proteins are determined how quick or slow the background develops. Two general classifications are silver amine or alkaline method and silver nitrate or acidic method (Rabilloud, 1990). The procedure for silver staining requires several steps includes fixing, washing, sensitizing, staining, developing and terminating. All of this step can be done in one day which requires 1-2 hours to get the protein bands. Thus, it is more time consuming but requires more steps with different solutions compare with CBB R staining that only require one to two steps.

### 3.0 Materials and method

#### 3.1 Study site

The root of the *Piper nigrum* sample was collected at Kampung Raeh near the Serian area which included healthy and disease root plant sample. The location of the sampling was the private agriculture farm which own by individual farmer. The healthy sample collected from the healthy growing plants of *P. nigrum* where it produces fruits and healthy green leaves. While the diseases roots sample was collected from the plants tree that showed symptoms of *Phytophthora capsici*, visibly stunted growth.

#### 3.2 Plant material

The root samples of the *P. nigrum* was collected and washed cleanly with running water to remove the soil attached with the roots. Then, the root samples were rinsed with distilled water and with 70% of ethanol to remove any remaining soil and other contamination. The samples were quickly stored at -80 °C to keep the freshness. The roots samples were finely powdered by grinding it with liquid nitrogen using mortar and pestle before continuing with extraction processes. This process is important to break the cell membrane and allow the movement of the solution.

### 3.3 First method of protein extraction: Phenol extraction

Based on Faurobert *et al.*, 2007, the steps was modified using 0.4 g of ground tissue that was suspended in 0.8 ml of extraction buffer in a centrifuge tube and vortexed to mix it. Then, it was incubated by shaking for 10 min on ice to limit the protease activity at low temperature. After that, an equal volume of Tris-buffered phenol was added, and the solution was vortexed and incubated on a shaker for another 10 min at room temperature. The sample was centrifuged to separate insoluble material, in the pellet, for aqueous and organic phases at 16 000 rcf for 10 min at 4 °C. The phenolic phase was seen on the top of the tube and pipette out carefully to avoid contact with the interphase and transfer into a new tube. Then, this phenol phase was back-extracted with 0.8 ml of extraction buffer. The sample was vortexed and shaken at room temperature for 10 min. Centrifugation for phase separation was repeated at 16 000 g for 10 min at 4 °C. The phenol phase seen on the top of the tube was carefully pipette out and transferred into a new tube (Faurobert *et al.*, 2007).

After that, three volume of 0.1 M ammonium acetate in cold methanol was added as precipitation solution. The tube was shaken by inverting, and the sample was incubated overnight at -20 °C. Protein was finally pelleted by centrifugation at 16 000 rcf for 10 min at 4 °C. The pellet was washed once with cooled precipitation solution and once with 80% cooled acetone. At each washing step, the sample was centrifuged at 16 000 rcf for 10 min at 4 °C. Finally, the pellet was air dry and to delay pellet resolubilization process, the protein pellet will be stored at -20 °C. (Faurobert *et al.*, 2007).



The simplified protocol from Rodrigues *et al.*, 2012, was used which representing considerable economy of reagents and time which had been modified from Wang *et al.*, 2006. The step was further modified in this experiment with increasing the used 0.4 g of ground powder from the roots sample of *P. nigrum*. The sample was resuspended in 0.8 ml of Tris-buffered phenol, pH 8.0 and 0.8 ml of SDS buffer that contained 0.1 M Tris-HCl, pH 8.0, 2% SDS, 5%  $\beta$ -mercaptoethanol, 30% sucrose, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The mixtures were vigorously vortexed and incubated for 10 min. Then, the mixtures were centrifuged at 16 000 rcf for 5 min at 4 °C. The top phenol layer was transferred into a new tube with the volume about 0.5 ml.

After that, three volumes of precipitation solution containing pre-cooled 0.1 M ammonium acetate in methanol was added into the tube and left overnight at -20 °C. The pellet obtained by centrifugation at 16 000 rcf for 5 min at 4 °C was washed same with phenol extraction method, once with cooled 0.1 M ammonium acetate in methanol and cooled 80% acetone. Finally, the pellet was let air dry and store at -20 °C (Rodrigues *et al.*, 2012).

### 3.5 Bradford assays

The protocols was adapted from Bradford, 1976, and some modification with the volume had been done from Stoscheck, 1990. The standard protein using BSA solution was prepared with different concentration for the calibration curve which was 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml and 1.0 mg/ml. Each of the volume was pipetted into 1.5 ml

Eppendorf tube and the volume was adjusted to 20  $\mu$ l by adding buffer solution, 0.05 M Tris-HCl. On the other hand, the sample protein was prepared with a range of 1, 1:1, 1:9 with the total of 20  $\mu$ l and pipetted into 1.5 ml Eppendorf tube. After that, all of the tube was added with 1 ml of Bradford reagent including the blank tube (20  $\mu$ l 0.05 M Tris-HCl), vortexed and incubated at room temperature for 5 min. Spectrophotometer (OPTIMA SP-300) was used to analyze the BSA standard solution and protein sample at absorbance of 595 nm (Bradford, 1976).

### **3.6 Bicinchinonic acid (BCA) assays**

The standard assay was adapted from Lovrien & Matulis, 1995, and Stoscheck, 1990, to prepare the solution and steps required. The BSA protein was used as the standard as the standard graph. Different concentration of BSA was prepared with 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml and 1.0 mg/ml into each of 1.5 ml Eppendorf tube and the volume was adjusted until 20  $\mu$ l using buffer solution (0.05 M Tris-HCl). For the protein sample, the dilution of 1:1 and 1:9 was prepared with adjusted volume of 20  $\mu$ l using buffer solution. After that, 1 ml of standard working solution was added to each sample and mixed using vortex. The samples then were incubated for 30 min at 60 °C. Next, the samples were let cool with running water before read at 562 nm using spectrophotometer (OPTIMA SP-300). The samples should be stable for at least one hour.

Protein extracts were separated using SDS-PAGE according to the protocol from Laemmli, 1970. The gel was prepared firstly with separation gels and then the stacking gels. After the stacking gels were hardened, the samples were loaded into the gels carefully with the loading of the ladder on the first lane. The gels were run in electrode buffer at 120 V for 45 min. When the running had finished, the gels were carefully handled to prevent the gels from breaking. The gels was washed and stained with staining solution, Coomassie Brilliant Blue (CBB) R or in fixing solution for silver staining before viewed. For the CBB-R staining, the gels were kept overnight. After that, destaining solution was added to the gels and incubated on shaker for 40 min and 15 min respectively before viewing the band.

### 3.7 Silver staining

The combined protocol Lin, 2012 and Schuchard *et al.*, n.d., was used for silver staining to detect the more protein sensitivity on the gel. After electrophoresis, the gel was placed in a tray with fixing solution (50% ethanol and 10% acetic acid) with constant agitation on a shaker for 20 min. The fixing solution was decanted and added with washing solution (30% ethanol) for 10 min with constant shaking. Then, rinsing solution made up with d H<sub>2</sub>O was replaced after washing solution had discarded and shakes for 10 min. After that, sensitizing solution (0.02% Sodium thiosulfate and 99.98% of d H<sub>2</sub>O) was replaced with constant agitation for 10 min. The sensitizing solution was poured out and added with rinsing solution twice to the gel for 10 min each and shakes with persistent speed. The gel was stained with 0.05 g silver nitrate in 50 ml d H<sub>2</sub>O for 10 min. Next, the staining solution

was replaced with rinsing solution for one minute and quickly added with developing solution (6% Sodium carbonate, 0.05% formalin (35% Formaldehyde), 0.0004% sodium thiosulfate) for 5-7 min where the gel was developed. The developing solution was discarded after the gel was fully developed and was terminated with 5% acetic acid for 5 min in constant speed.